REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and following remarks.

Claim 29 has been amended to remove the species of viral hepatitis. New claim 36 is added to recite the species of viral hepatitis.

In view of the foregoing, the rejection of claim 29 under 35 USC 112, second paragraph is deemed to be overcome.

Claims 25-35 are rejected under 35 USC 103 as being unpatentable over Sakai et al. This ground of rejection is respectfully traversed.

The Examiner reasons that Sakai et al. disclose that 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol (hereinafter referred to as the present compound) is useful for the treatment of hepatitis B (i.e., viral infection caused by hepatitis B virus in the liver), and therefore, it would have been reasonably expected that the present compound would be effective for the treatment of viral myocarditis (viral infection caused by the same hepatitis B virus in the heart muscle) and further that the treatment of infection by hepatitis B virus would lead to the amelioration of viral cytotoxicity caused by the same virus.

Such reasoning is respectfully submitted to be incorrect for the following reasons.

Sakai et al. teach that the present compound is useful for the treatment or prevention of hepatic diseases such as acute liver necrosis (e.g., necrosis caused by toxins, viral hepatitis, shock or anoxia), viral hepatitis B, non-A/non B hepatitis and cirrhosis, because it has a liver regenerating activity and/or an activity of promoting hypertrophy and hyperplasia of hepatocytes. See column 5, lines 28-40. In other words, the description of the usefulness of the present compound against viral hepatitis in Sakai et al. is based on <u>the liver regenerating activity</u> and <u>activity of promoting hypertrophy and hyperplasia of hepatocytes</u> of the present compound. Therefore, whether or not the present compound is effective for the diseases caused by hepatitis virus <u>in the heart muscle</u> is not suggested by Sakai et al.

In addition, the Examiner points out that Sakai et al. teach the usefulness of the present compound for the treatment of necrosis induced by toxins. A reference to the portion the

Examiner points out reveals that it exemplifies acute liver necrosis as evidenced by the description, "acute liver necrosis (e.g., necrosis caused by toxins, viral hepatitis, shock or anoxia)". However, this description does not suggest the usefulness of the present compound against viral cytotoxicity in an organ other than the liver.

The effect of the present invention on viral myocarditis and viral cytotoxicity has been clearly confirmed in disease models infected with the virus. See the Experimental Examples in the present specification. Such effect of the present compound against viral myocarditis and viral cytotoxicity would be unexpected by those of ordinary skill in the art from Sakai et al., which merely teaches usefulness of the compound against hepatic diseases based on a liver regenerating activity and an activity of promoting hypertrophy and hyperplasia of hepatocytes.

The therapeutic effect of the present compound against viral myocarditis has been first found by the present inventor. This effect has been reported in the enclosed article, published in the Journal of the American College of Cardiology, Vol. 37, No. 6, May 2001, pp. 1713-1718. Please note that "FTY720" and "FTY" refer to 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride. See page 1713, left column line 3 from the bottom - the last line.

The Examiner further reasons that the use of the present compound for the treatment of viral infection caused by an RNA virus such as picornavirus would have been reasonably expected, since treatment of viral infection caused by RNA viruses such as hepatitis B virus, non-A, and non B hepatitis virus by the use of the present compound was known from Sakai et al.

However, hepatitis B virus is a DNA virus. Therefore, the description relating to hepatitis B virus in Sakai et al. does not suggest the effect for the treatment of viral myocarditis or viral diseases induced by viral myocarditis caused by an RNA virus.

The Examiner also points out the teaching of Sakai et al. that the present compound is useful for the treatment of infectious diseases caused by pathogenic microorganisms. See column 4, lines 27-28. However, pathogenic microorganisms encompass a wide variety of microorganisms such as bacteria, viruses, fungi and the like. Sakai et al. do not teach a confirmed effect of the present compound against viral myocarditis or viral cytotoxicity. As mentioned earlier, the effect of the present compound on viral myocarditis and viral cytotoxicity has been

first confirmed by the present inventor in disease models infected with the virus. The therapeutic effect and viral cytotoxicity-ameliorating effect on viral myocarditis afforded by the present compound cannot be reasonably expected by those of ordinary skill in the art from Sakai et al.

As the Examiner will appreciate, the pharmaceutical art is unpredictable. The effectiveness of a particular compound against a specific disease condition cannot be predicted until tests are conducted to confirm or deny the utility of the compound. The teachings of Sakai et al. are vague and general, and are directed to different diseases than the claimed diseases. One skilled in the art could not have had a reasonable expectation from Sakai et al. that the present compound would be useful in treating viral myocarditis or viral cytotoxicity.

In view of the foregoing, it is respectfully submitted that the application is now in condition for allowance, and such allowance is solicited.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made**."

Respectfully submitted,

Akira MATSUMORI

By: Warren Cheek Jr

Warren M. Cheek, Jr.
Registration No. 33,367
Attorney for Applicant

WMC/dlk
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
April 8, 2003

26. **(New)** A method for the prophylaxis or treatment of viral diseases induced by viral myocarditis, which comprises administering an effective amount of 2-amino-2-(2-(4-octylphenyl)ethyl)propane-1,3-diol or a pharmacologically acceptable salt thereof to a patient in need thereof.

27. **(New)** The method of claim 25, wherein the viral myocarditis is caused by RNA virus or hepatitis virus.

28. **(New)** The method of claim 27, wherein the RNA virus is orthomyxovirus or picornavirus.

Amended

29. ~~**(New)**~~ The method of claim 26, wherein the viral disease is viral hepatitis ~~(type A, type B, type C, type E, type G and type TTV)~~, adenovirus infection, influenza, herpes infection, viral encephalitis, cytomegalovirus infection, viral enteritis or viral pericarditis.

30. **(New)** A method for the amelioration or prophylaxis of viral cytotoxicity, which comprises administering an effective amount of 2-amino-2-(2-(4-octylphenyl)ethyl)propane-1,3-diol or a pharmacologically acceptable salt thereof to a patient in need thereof.

31. **(New)** The method of claim 26, wherein the viral diseases induced by viral myocarditis are caused by RNA virus or hepatitis virus.

32. **(New)** The method of claim 31, wherein the RNA virus is orthomyxovirus or picornavirus.

33. **(New)** A method for the prophylaxis or treatment of viral myocarditis, which comprises administering an effective amount of 2-amino-2-(2-(4-octylphenyl)ethyl)propane-1,3-diol hydrochloride to a patient in need thereof.

Journal of the American College of Cardiology
Vol. 37, No. 6, 2001
ISSN 0735-1097/01/$20.00
Published by Elsevier Science Inc.
PII S0735-1097(01)01204-9

EXPERIMENTAL STUDIES

Therapeutic Effects of FTY720, a New Immunosuppressive Agent, in a Murine Model of Acute Viral Myocarditis

Tadashi Miyamoto, MD, Akira Matsumori, MD, PhD, FACC, Myung-Woo Hwang, MD, PhD, Ryosuke Nishio, MD, PhD, Haruyasu Ito, MD, Shigetake Sasayama, MD, PhD, FACC

Kyoto, Japan

OBJECTIVES This study examines the efficacy of FTY720 (FTY), a new immunosuppressor, in the treatment of acute viral myocarditis in a murine model.

BACKGROUND Immunosuppressive agents have no proven therapeutic efficacy in experimental or clinical myocarditis.

METHODS Encephalomyocarditis virus was inoculated i.p. in DBA/2 mice on day 0. Postinoculation treatment consisted of FTY 10 mg/kg/day p.o. (FTY group), or cyclosporine A (CsA) 40 mg/kg/day p.o. (CsA group) or distilled water p.o. only (control group). Survival until day 14, as well as cardiac histopathology, virus concentrations, cytokines (interleukin [IL]-2, IL-12, interferon [IFN]-gamma and tumor necrosis factor [TNF]-alpha) and nitric oxide (NO) on day 5 were examined.

RESULTS In the control and CsA groups, all mice died within 10 and 7 days, respectively. However, in the FTY group, 27% of the animals survived up to day 14. Compared with the control group, 1) histological scores were significantly lower in the FTY group but unchanged in the CsA group; 2) virus concentration was significantly higher in the CsA group but not in the FTY group; 3) expressions of IL-2, IL-12 and IFN-gamma in the heart were suppressed in both the FTY and CsA groups, though suppression was weaker in the FTY group; 4) TNF-alpha and NO were significantly increased in the CsA group but not in the FTY group.

CONCLUSIONS FTY720 had a significant therapeutic effect in acute experimental myocarditis without inducing excessive virus replication. This report is the first to describe a beneficial effect by an immunosuppressive agent in the treatment of acute viral myocarditis. (J Am Coll Cardiol 2001;37:1713–8) © 2001 by the American College of Cardiology

Recent developments in the molecular analysis of autopsy and biopsy specimens have clarified the relationship between viral myocarditis and dilated cardiomyopathy (1,2). Therefore, treatments targeted toward viral infections are becoming important in the prevention of heart failure complicating myocarditis. However, despite intensive experimental and clinical research, specific therapies remain to be developed. Because both the direct cytopathic effects of the virus and the host immune response that it induces appear to be responsible for the manifestations of viral myocarditis (3), therapeutic effects have been expected from immunosuppression, a hypothesis supported by anecdotal, isolated case reports. However, treatment of myocarditis with immunosuppressors such as corticosteroids (4,5), cyclosporine A (CsA) (6–8) or FK506 (tacrolimus) (9) has been ineffective experimentally and in the recent clinical Myocarditis Treatment Trial (10).

The new synthetic immunosuppressor FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride, FTY), a derivative of ISP-I (myriocin) isolated from the fungus *Isaria sinclairii* (11,12), has unique properties, unlike those of CsA and FK506 or of corticosteroids (13). Its precise mechanisms of action remain unclear, though it has been shown to accelerate the sequestration of circulating mature lymphocytes into lymph nodes and Peyer's patches (14) and to decrease the number of peripheral blood lymphocytes and their infiltration into target tissues (15). In contrast to CsA and FK506, FTY does not suppress the proliferative response of T cells (16) or the production of IL-2 by lymphocytes in vitro (14). In experimental allogenic transplantation models of skin (14,17,18), liver (18), kidney (19) and heart (19,20), FTY has already been found as effective as, or more effective than, CsA in promoting the survival of allografts. Despite its importance in organ transplantation, the efficacy of FTY against infectious pathogens has been the object of only a few reports (21).

This study was performed to examine the efficacy of FTY in the treatment of experimental acute viral myocarditis induced by encephalomyocarditis virus (EMCV) in a murine model.

From the Department of Cardiovascular Medicine, Kyoto University, Kyoto, Japan. This work was supported in part by a research grant from the Japanese Ministry of Health and Welfare, and by a grant-in-aid for general scientific research from the Japanese Ministry of Education, Science and Culture.

Manuscript received October 10, 2000; revised manuscript received January 12, 2001, accepted January 29, 2001.

MATERIALS AND METHODS

Pharmaceuticals. FTY, supplied as dry powder by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan), was

Abbreviations and Acronyms

CsA	= cyclosporine A
EMCV	= encephalomyocarditis virus
FTY	= FTY720
IFN	= interferon
iNOS	= inducible NO synthase
NO	= nitric oxide
TNF	= tumor necrosis factor

dissolved in sterile distilled water. Cyclosporine A was purchased from Sigma Pharmaceuticals (St. Louis, Missouri) and dissolved in olive oil (20).

Experimental viral myocarditis. Twenty-eight-day-old male DBA/2 mice were purchased from Shizuoka Agricultural Cooperation Association (Shizuoka, Japan) and housed in a special pathogen-free animal facility of the Kyoto University Hospital. The experiments were performed in a well-established murine model of EMCV-induced myocarditis (22). Briefly, the mice were inoculated intraperitoneally with 10 plaque-forming units (pfu) of the myocardiotrophic (M) variant of EMCV on day 0 and observed daily. Starting on day 0, FTY was daily administered orally in a dose of 10 mg/kg/day in one group of mice (FTY group), CsA was daily administered orally in a dose of 40 mg/kg/day in an other group (CsA group) and control mice received distilled water only (control group) (20). Each drug, or the distilled water, was administered via a gastric tube to guarantee reliable delivery. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Survival study. In the survival experiments, the animals (nine in the CsA group, 21 in the FTY group, 22 in the control group) were observed daily between day 0 and day 14. In another series of experiments, the same doses of CsA or FTY were administered to uninfected mice (five in each group) in order to examine the effects of the drugs on survival in absence of infection.

Histopathologic examination. For histopathologic studies, hearts from surviving mice (six in the CsA group, eight in the FTY group, nine in the control group) were harvested on day 5, fixed in 10% formalin and embedded in paraffin. The ventricle was sliced transversally, stained with hematoxylin and eosin, and examined by light microscopy. Extent of cellular infiltration and myocardial necrosis were graded as follows: 0 = no lesion; 1+ = lesions involving <25% of the myocardium; 2+ = lesions involving 25% to 50% of the myocardium; 3+ = lesions involving 50% to 75% of the myocardium; 4+ = lesions involving >75% of the myocardium. Extent of infiltration and necrosis was scored blindly by two independent trained observers, whose scores were averaged. The details of the method have been described previously (23). In another series of experiments, the same doses of CsA or FTY were administered to uninfected mice (five in each group) to examine the effects of the drugs on histopathology in absence of infection.

Intramyocardial virus concentration. For the measurements of intramyocardial virus concentration, the ventricles from surviving mice (five in the CsA group, seven in the FTY group, eight in the control group) were harvested aseptically on day 5, weighed, and homogenized in 1 ml of phosphate buffered saline. After centrifugation at 14,000 rpm for 20 min at 4°C, 0.1 ml of supernatant was inoculated into human amnion FL cell monolayers for 60 min at 37°C in 5% CO_2. The cells were overlaid with 3 ml of medium containing 4% fetal calf serum and 1% methylcellulose. After 20 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the cells were fixed with acetic acid and methanol (in a ratio of 1:2) and stained with 1% crystal violet, and the plaques were counted under an inverted microscope (23). If the plaques were too numerous to count, the assay was repeated after appropriate dilution of the supernatants with Dulbecco's modified Eagle's medium (GIBCO BRL, New York, New York). Each value represents the average of two experiments. Myocardial virus concentration is expressed as plaque forming units/g of heart.

Intracardiac cytokine assay. For assays of intracardiac cytokine, the ventricles from surviving mice (six in the CsA group, seven in the FTY group, eight in the control group) were harvested on day 5, homogenized in 1 ml PBS solution with an ultrasonic homogenizer (Astrason Ultrasonic Liquid Processor, model XL2020, MISONIX Inc., Farmingdale, New York) and centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was used for the assay of IL-2, IL-12, IFN-gamma and TNF-alpha. Each cytokine protein level was measured by enzyme-linked immunosorbent assay (24) with commercially available kits according to each manufacturer's instructions. Enzyme-linked immunosorbent assay kits for mouse IL-2 and IFN-gamma were purchased from GENZYME Corporation (Cambridge, Massachusetts) and kits for mouse IL-12 and TNF-alpha from ENDOGEN Inc. (Cambridge, Massachusetts).

The total protein concentration in each supernatant was also measured by the bicinchoninate acid method, and the ratio of cytokine concentration/total protein concentration was calculated (25). Each cytokine protein level was expressed as pg or ng/mg total protein.

Intracardiac nitric oxide assay. Intracardiac nitric oxide (NO) content was measured by modified Griess assay in the same supernatants as the intracardiac cytokines (25,26). In brief, 50 μl of supernatant or standard nitrite was mixed with 10 μl of 10 μM beta-NADPH, and 40 μl premixed master mix (500 μM glucose-6-phosphate, 160 U/l glucose-6-phosphate dehydrogenase, 80 U/l nitrate reductase, 0.2 mM phosphate buffer) was added and incubated for 45 min at 20°C. Subsequently, 50 μl of 1% sulfanilamide in 5% H_3PO_4 and 50 μl of 0.1% naphthylethylene diamine dihydrochloride were also added and incubated for 10 min at 20°C. The optical density was measured at 540 nm with a microplate reader. Nitrite concentration in each sample was calculated from standards. Measurements of all samples

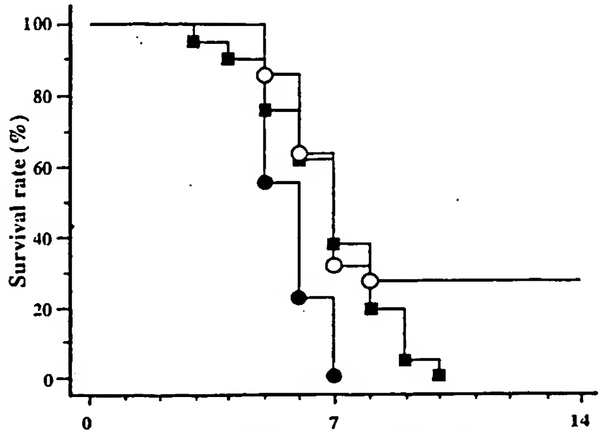

Figure 1. Fourteen-day cumulative survival after encephalomyocarditis virus inoculation in FTY720 treated mice (open circles), versus cyclosporine A-treated mice (filled circles) versus control mice (filled squares).

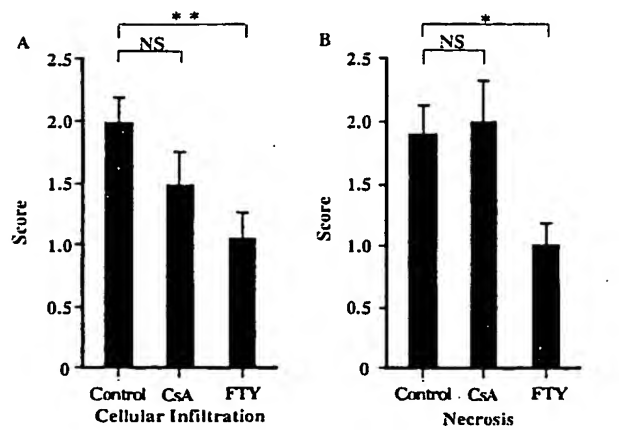

Figure 2. Myocardial histopathologic scores for cellular infiltration (A) and necrosis (B) on day 5 after encephalomyocarditis virus inoculation in surviving mice. *$p < 0.05$; **$p < 0.01$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

and standards were made in duplicate. The NO content in the heart was determined by dividing each nitrite concentration by the total protein concentration in each supernatant, expressed as μM/mg protein.

Statistical analysis. Statistical analysis of survival rates was performed by the Kaplan-Meier method, followed by Fisher's protected least significant difference. Histopathological scores; intracardiac virus concentrations; intracardiac IL-2, IL-12, IFN-gamma and TNF-alpha protein levels and NO content were compared by one-way ANOVA, followed by Fisher's protected least significant difference. A value of $p < 0.05$ was considered significant. Data are expressed as mean ± SEM.

RESULTS

Effects of FTY720 and CsA on survival in EMCV-infected mice. In the control group all mice died within 10 days. Cyclosporine A had a negative effect on survival rate, because none of the mice in that group survived past day 7 (Fig. 1), a significantly worse outcome than in the control group. In contrast, six of 22 mice (27%) treated with FTY were alive on day 14 ($p = 0.051$ vs. control, and $p = 0.0128$ vs. CsA). In the experiments performed in uninfected mice, all animals survived until day 14 after the administration of either drug (data not shown).

Histopathologic examinations. In the CsA group, the cellular infiltration score was lower, and the necrosis score slightly higher than in the control group (1.50 ± 0.26 vs. 2.00 ± 0.19 for infiltration, and 2.00 ± 0.32 vs. 1.89 ± 0.23 for necrosis), though these differences were not statistically significant. In contrast, the scores of both cellular infiltration and necrosis in the FTY group were significantly lower than in the control group (1.06 ± 0.19 vs. 2.00 ± 0.19 for infiltration and 1.00 ± 0.19 vs. 1.89 ± 0.23 for necrosis, Figs. 2 and 3). In the uninfected mice, neither drug had an apparent effect on the cardiac histology on day 5 (data not shown).

Intramyocardial virus concentration. Cyclosporine A increased virus replication in the heart 20-fold compared with control measurements ($9.94 \pm 6.30 \times 10^7$ vs. $5.18 \pm 2.73 \times 10^6$, Fig. 4). In contrast, FTY had no such effect on virus replication ($1.23 \pm 0.53 \times 10^7$ vs. $5.18 \pm 2.73 \times 10^6$, Fig. 4).

Measurements of intracardiac IL-2, IL-12, IFN-gamma and TNF-alpha. The effects of treatment with FTY and CsA on intramyocardial cytokines expression are summarized in Figure 5. Consistent with previous observations (27), protein levels of intracardiac IL-2, known to be associated with T-cell proliferation, were lower in both the CsA and the FTY groups than in the control group. However, the suppression of IL-2 in the FTY group was less prominent than in the CsA group. Protein levels of intracardiac IL-12, a Th1-specific cytokine (28), were

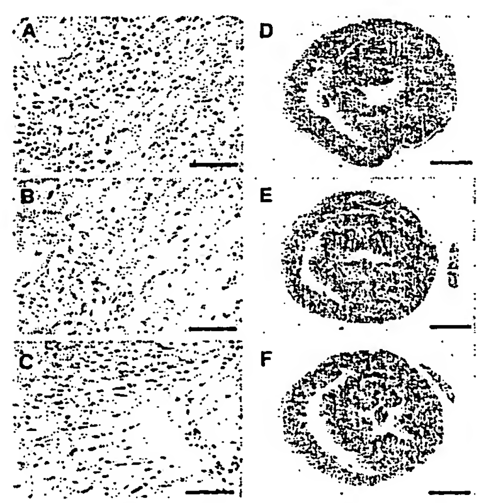

Figure 3. Representative photomicrograph of histopathologic sections of the heart. A and D: control mouse; B and E: cyclosporine A-treated mouse; C and F: FTY720-treated mouse. Hematoxylin and eosin stain, A,B,C: original magnification ×200, bar = 50 μm, D,E,F: original magnification ×2, bar = 1 mm.

JACC Vol. 37, No. 6, 2001
May 2001:1713–8

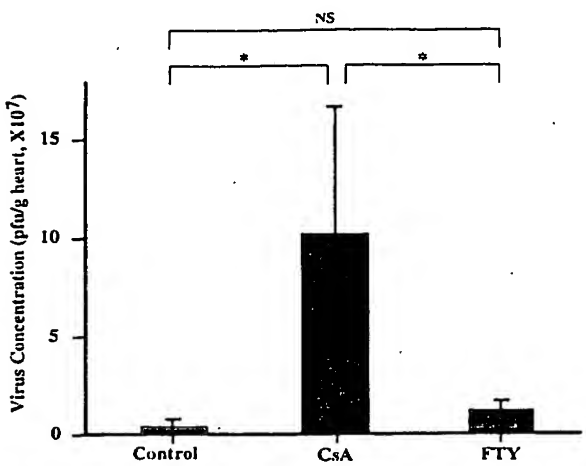

Figure 4. Effects of FTY (FTY720) and cyclosporine A (CsA) on intracardiac virus concentration on day 5 after encephalomyocarditis virus inoculation in surviving mice. *$p < 0.05$. NS = not significant.

significantly lower in the CsA group than in both the FTY and the control groups. The difference between the FTY and control groups was also statistically significant. Similarly, intracardiac IFN-gamma, which inhibits virus replication (29), was markedly decreased in the CsA group and only mildly suppressed in the FTY compared with the control group. Conversely, concentrations of intracardiac

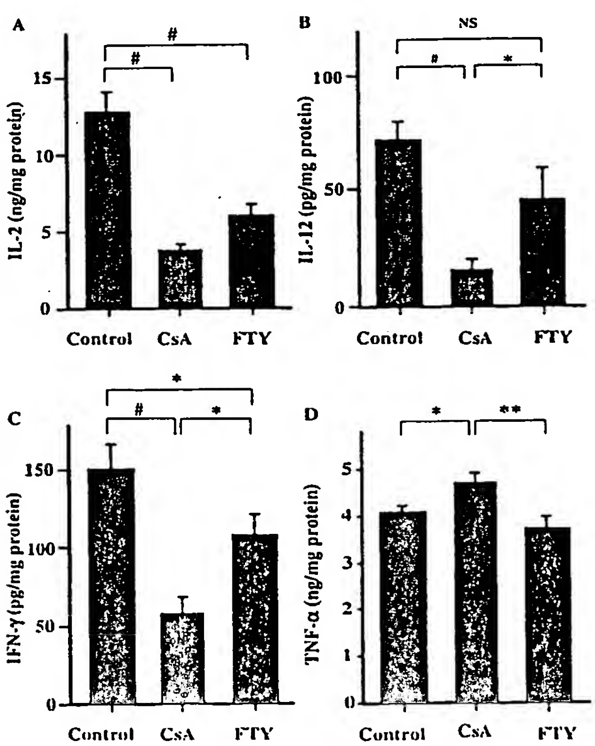

Figure 5. Intracardiac cytokine protein levels on day 5 after encephalomyocarditis virus inoculation in surviving mice. A: interleukin (IL)-2; B: IL-12; C: interferon (IFN)-gamma; D: tumor necrosis factor (TNF)-alpha. *$p < 0.05$, **$p < 0.01$, # $p < 0.001$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

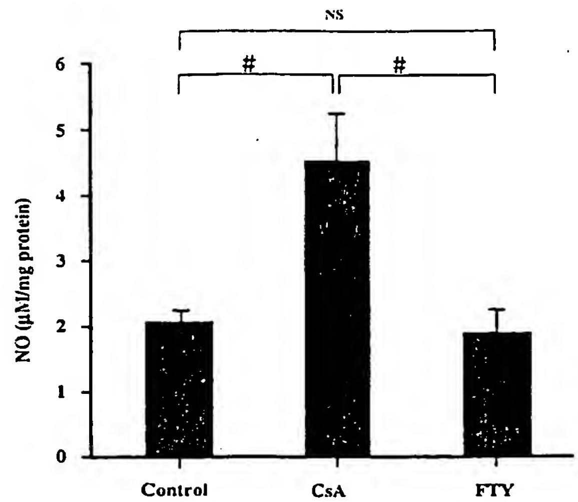

Figure 6. Intracardiac nitric oxide (NO) content on day 5 after encephalomyocarditis virus inoculation in surviving mice. #$p < 0.001$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

TNF-alpha, one of the proinflammatory cytokines, were increased in the CsA group, but not in the FTY group, compared with control.

Intracardiac NO content. In parallel with the changes observed in TNF-alpha, intracardiac NO content was significantly increased in the CsA group, but not in the FTY group, compared with control (Fig. 6).

DISCUSSION

Superiority of FTY720 over other immunosuppressors in viral myocarditis. This study showed that the new immunosuppressor FTY had notable therapeutic effects in this murine model of viral myocarditis, by a prolongation of survival and attenuation of histologic abnormalities. These effects were exerted by gentle cardiac immunosuppression, without the induction of excessive virus replication, despite the use of relatively high doses of FTY. The doses of CsA administered were also high because of its peculiar pharmacokinetics in mice (30,31). However, a detrimental effect of CsA in EMCV-induced myocarditis has been observed in other studies in which lower doses of the drug were used (6,7).

FTY is reported to be effective in a myosin-induced autoimmune myocarditis rat model (32). However, another immunosuppressor, FK506, which is ineffective in a viral myocarditis murine model (9), is also known to prevent the progression of myosin-induced autoimmune myocarditis in a rat model (33,34). A general complication associated with the use of immunosuppressors in the midst of a viral infection is uncontrolled virus replication (4,6,35,36), so the two models of myocarditis should be distinguished in terms of immunosuppressive therapy. In this study, virus concentrations in the heart were significantly increased by CsA, compared with control, as previously reported (4). This detrimental effect was not observed with FTY.

Characteristic effects of FTY on the expression of cardiac cytokines, on NO and on virus replication. To investigate the mechanism of this superior effect of FTY, we measured several cytokines associated with cell-mediated immunity, and one major proinflammatory cytokine. Interleukin-2 activates T lymphocyte proliferation, the suppression of which, at a transcriptional level, is known to be mediated by calcineurin-related immunosuppressant such as CsA and FK506 (13). In this study, as expected, CsA prominently suppressed the intracardiac expression of IL-2. In addition, both IL-12, which promotes differentiation to the Th1 cell subset (37), and IFN-gamma, a Th1 cytokine with antiviral activity, were markedly suppressed in the hearts of CsA-treated mice. This direct inhibition of T cell-mediated immunity may explain the observed accelerated virus replication. FTY also suppressed the expression of these three cytokines in the heart, though to a considerably lesser degree than CsA. The most likely proposed immunosuppressive mechanisms of FTY are an accelerated sequestration of circulating mature lymphocytes into lymph nodes and Peyer's patches (14), and a decrease in peripheral blood lymphocytes and infiltration into target tissues (15). In addition, FTY does not directly suppress the proliferative responses of T cells (16) or the production of IL-2 by lymphocytes in vitro (14). These mechanisms of action explain the absence of accelerated virus replication associated with the FTY treatment. Furthermore, intracardiac TNF-alpha and NO were proportionally elevated in the CsA group compared with the control group. Cyclosporine A is known to suppress TNF-alpha production from lymphocytes but not from macrophages in vitro (38). Immunohistochemical analyses have shown TNF-alpha staining in macrophages within the heart affected by EMCV-induced myocarditis (39). In addition, TNF-alpha mRNA was also induced in purified neonatal murine cardiac fibroblasts infected with EMCV in vitro (unpublished data), TNF-alpha concentrations in plasma and in the heart were increased in EMCV-induced myocarditis (23,25) and treatment with anti-TNF-alpha antibody had a therapeutic effect in this model (23). From these observations combined, one may hypothesize that excessive viral replication directly induced TNF-alpha in the hearts of CsA-treated mice. Nitric oxide is produced by inducible NO synthase (iNOS), which is induced by proinflammatory cytokines, including TNF-alpha. Nitric oxide not only has antiviral effects but also causes myocardial injury. Immunomodulating drugs, which suppress proinflammatory cytokines and iNOS (40–42), are therapeutic in EMCV-induced myocarditis (25). Increases in TNF-alpha and NO concentrations may also explain, at least in part, the worsening of heart failure associated with CsA treatment. In contrast, in our study, FTY did not increase the concentrations of TNF-alpha or NO.

Opportunistic infection is the most troublesome complication of immunosuppression in organ transplantation (35). In this respect, our results suggest that FTY may be superior to the other immunosuppressors, although its efficacy in the presence of common opportunistic infectious pathogens needs to be examined.

Study limitations. In a clinical situation, treatment of viral myocarditis cannot be initiated at the very onset of infection. Further experiments, designed with a delay between virus inoculation and onset of treatment, need to be performed. This study, nevertheless, remains noteworthy for having observed no increase in virus replication from treatment with FTY. In addition, further studies of the specific therapeutic effects of FTY are needed, as its precise pharmacologic properties remain incompletely understood.

In conclusion, this report is the first to describe a therapeutic effect by an immunosuppressive agent in the treatment of acute viral myocarditis.

Acknowledgments
We would like to express our gratitude to Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan), for kindly supplying FTY, and to Ms. S. Sakai for preparing the manuscript.

Reprint requests and correspondence: Dr. Akira Matsumori, Department of Cardiovascular Medicine, Kyoto University, 54 Kawaracho Shogoin, Sakyo-ku, Kyoto 606-8397, Japan. E-mail: amat@kuhp.kyoto-u.ac.jp.

REFERENCES

1. Kawai C. From myocarditis to cardiomyopathy: mechanisms of inflammation and cell death: learning from the past for the future. Circulation 1999;99:1091–100.
2. Matsumori A. Molecular and immune mechanisms in the pathogenesis of cardiomyopathy—role of viruses, cytokines, and nitric oxide. Jpn Circ J 1997;61:275–91.
3. Huber SA, Lodge PA. Coxsackievirus B-3 myocarditis in Balb/c mice. Evidence for autoimmunity to myocyte antigens. Am J Pathol 1984; 116:21–9.
4. Tomioka N, Kishimoto C, Matsumori A, Kawai C. Effects of prednisolone on acute viral myocarditis in mice. J Am Coll Cardiol 1986;7:868–72.
5. Herzum M, Ruppert V, Kuytz B, Jomma H, Nakamura I, Maisch B. Coxsackievirus B3 infection leads to cell death of cardiac myocytes. J Mol Cell Cardiol 1994;26:907–13.
6. Monrad ES, Matsumori A, Murphy JC, Fox JG, Crumpacker CS, Abelmann WH. Therapy with cyclosporine in experimental murine myocarditis with encephalomyocarditis virus. Circulation 1986;73: 1058–64.
7. O'Connell JB, Reap EA, Robinson JA. The effects of cyclosporine on acute murine coxsackie B3 myocarditis. Circulation 1986;73:353–9.
8. Herzum M, Huber SA, Weller R, Grebe R, Maisch B. Treatment of experimental murine coxsackie B3 myocarditis. Eur Heart J 1991;12 Suppl D:200–1.
9. Matsumori A. Animal models: pathological findings and therapeutic considerations. In: Banatvala JE, ed. Viral infections of the heart. 1st ed. London: Edward Arnold, 1993:110–37.
10. Mason JW, O'Connell JB, Herskowitz A, et al. A clinical trial of immunosuppressive therapy for myocarditis. The Myocarditis Treatment Trial Investigators. N Engl J Med 1995;333:269–75.
11. Fujita T, Yoneta M, Hirose R, Sasaki S, Arita M, Chiba K. Simple compounds, 2-substituted-2-amino-1, 3-propanediols have potent immunosuppressive activity. Bioorg Med Chem Lett 1995;5:847–52.
12. Adachi K, Kohara T, Arita M, Chiba K, Sasaki S, Fujita T. Design, synthesis, and structure-activity relationships of 2-substituted-2-

JACC Vol. 37, No. 6, 2001
May 2001:1713–8

amino-1, 3-propanodiols: discovery of a novel immunosuppressant, FTY720. Bioorg Med Chem Lett 1995;5:853–6.

13. Suthanthiran M, Morris RE, Strom TB. Immunosuppressants: cellular and molecular mechanisms of action. Am J Kidney Dis 1996;28: 159–72.
14. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol 1998;160:5037–44.
15. Yanagawa Y, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats, III. Increase in frequency of CD62L-positive T cells in Peyer's patches by FTY720-induced lymphocyte homing. Immunology 1998;95:591–4.
16. Luo Z-J, Tanaka T, Kimura F, Miyasaka M. Analysis of the mode of action of a novel immunosuppressant FTY720 in mice. Immunopharmacology 1999;41:199–207.
17. Chiba K, Hoshino Y, Suzuki C, et al. FTY720, a novel immunosuppressant possessing unique mechanisms: I. Prolongation of skin allograft survival and synergistic effect in combination with cyclosporin in rats. Transplant Proc 1996;28:1056–69.
18. Suzuki S, Enosawa S, Kakefuda T, et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. Transplantation 1996; 61:200–5.
19. Suzuki S. FTY720: mechanisms of action and its effect on organ transplantation. Transplant Proc 1999;31:2779–82.
20. Hwang MW, Matsumori A, Furukawa Y, et al. FTY720, a new immunosuppressant, promotes long-term graft survival and inhibits the progression of graft coronary artery disease in a murine model of cardiac transplantation. Circulation 1999;100:1322–9.
21. Brinkmann V, Pinschewer D, Feng L. FTY720 suppresses immune response by modulating G-protein coupled receptors on lymphocytes resulting in altered lymphocyte homing. Transplantation 1999:S226.
22. Matsumori A, Kawai C. An experimental model for congestive heart failure after encephalomyocarditis virus myocarditis in mice. Circulation 1982;65:1230–5.
23. Yamada T, Matsumori A, Sasayama S. Therapeutic effect of anti-tumor necrosis factor-alpha antibody on the murine model of viral myocarditis induced by encephalomyocarditis virus. Circulation 1994; 89:846–51.
24. Nishio R, Matsumori A, Shioi T, Ishida H, Sasayama S. Treatment of experimental viral myocarditis with interleukin-10. Circulation 1999; 100:1102–8.
25. Iwasaki A, Matsumori A, Yamada T, et al. Pimobendan inhibits the production of proinflammatory cytokines and gene expression of inducible nitric oxide synthase in a murine model of viral myocarditis. J Am Coll Cardiol 1999;33:1400–7.
26. Verdon CP, Burton BA, Prior RL. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. Anal Biochem 1995;224:502–8.
27. Yanagawa Y, Sugahara K, Kataoka H, Kawaguchi T, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. J Immunol 1998;160:5493–9.
28. Shioi T, Matsumori A, Nishio R, Ono K, Kakio T, Sasayama S. Protective role of interleukin-12 in viral myocarditis. J Mol Cell Cardiol 1997;29:2327–34.
29. Matsumori A, Kawai C, Crumpacker CS, Abelmann WH. Pathogenesis and preventive and therapeutic trials in an animal model of dilated cardiomyopathy induced by a virus. Jpn Circ J 1987;51:661–4.
30. Kroczek RA, Black CD, Barbet J, Shevach EM. Mechanism of action of cyclosporin A in vivo. I. Cyclosporin A fails to inhibit T lymphocyte activation in response to alloantigens. J Immunol 1987;139:3597–603.
31. Batiuk TD, Urmson J, Vincent D, Yatscoff RW, Halloran PF. Quantitating immunosuppression. Estimating the 50% inhibitory concentration for in vivo cyclosporine in mice. Transplantation 1996; 61:1618–24.
32. Kitabayashi H, Isobe M, Watanabe N, Suzuki J, Yazaki Y, Sekiguchi M. FTY720 prevents development of experimental autoimmune myocarditis through reduction of circulating lymphocytes. J Cardiovasc Pharmacol 2000;35:410–6.
33. Hanawa H, Kodama M, Zhang S, Izumi T, Shibata A. An immunosuppressant compound, FK-506, prevents the progression of autoimmune myocarditis in rats. Clin Immunol Immunopathol 1992;62: 321–6.
34. Kodama M, Hanawa H, Zhang S, et al. FK506 therapy of experimental autoimmune myocarditis after onset of the disease. Am Heart J 1993;126:1385–92.
35. Macdonald PS, Keogh AM, Marshman D, et al. A double-blind placebo-controlled trial of low-dose ganciclovir to prevent cytomegalovirus disease after heart transplantation. J Heart Lung Transplant 1995;14:32–8.
36. Maisch B, Herzum M, Hufnagel G, Schonian U. Immunosuppressive and immunomodulatory treatment for myocarditis. Curr Opin Cardiol 1996;11:310–24.
37. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787–93.
38. Williams RO, Mauri C, Mason LJ, et al. Therapeutic actions of cyclosporine and anti-tumor necrosis factor alpha in collagen-induced arthritis and the effect of combination therapy. Arthritis Rheum 1998;41:1806–12.
39. Shioi T, Matsumori A, Sasayama S. Persistent expression of cytokine in the chronic stage of viral myocarditis in mice. Circulation 1996;94: 2930–7.
40. Matsumori A, Ono K, Sato Y, Shioi T, Nose Y, Sasayama S. Differential modulation of cytokine production by drugs: implications for therapy in heart failure. J Mol Cell Cardiol 1996;28:2491–9.
41. Matsumori A, Okada I, Shioi T, et al. Inotropic agents differentially inhibit the induction of nitric oxide synthase by endotoxin in cultured macrophages. Life Sci 1996;59:L121–5.
42. Matsumori A. The use of cytokine inhibitors. A new therapeutic insight into heart failure. Int J Cardiol 1997;62 Suppl 1:S3–12.